Delineating Role of Small Extracellular Vesicles associated Protein Signature with Breast Cancer Chemoresistance

PhD Synopsis

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Background

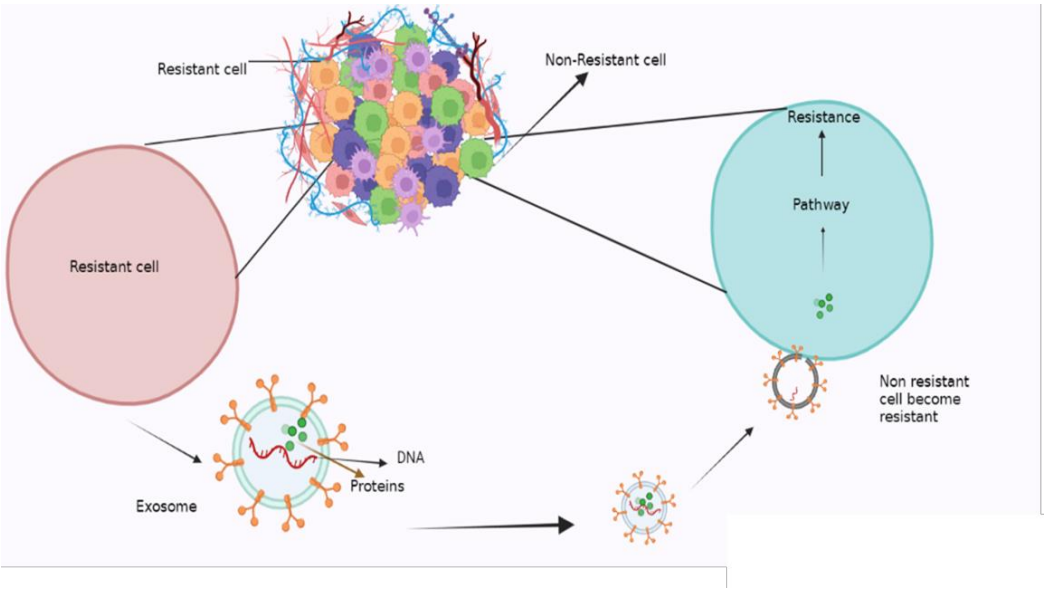
Cancer has exceeded all other genesis of death and is paramount as the leading cause of mortality worldwide, with a total of 9,958,133 confirmed deaths and 19,292,786 new cases (Siegel *et al.,* 2021). Breast cancer (BC) is the most prevalent malignancy and the 2nd leading cause of cancer-related death in women (Ferlay *et al.,* 2019). In India Breast cancer occurs at earlier age in women and patients usually present with advance stage of disease. Besides, breast cancer is the most common cancer in cities and 2nd most common in the rural areas. Studies indicate that as India becomes westernized, the incidence rate for breast cancer increases. Breast cancer is a variegated disease with multiple distinct subtypes that differ in anatomy, genetics, and clinical behavior and remains one of the primary causes of cancer related deaths among females worldwide. It is a very heterogeneous disease with more than 20 distinct subtypes that differ in morphology, genetics, and clinical behavior. Based on gene expression, B.C. is classified into luminal A, luminal B, HER2-enriched, and triple-negative breast cancer (TNBC) subtypes (Jenkins *et al.*, 2021). Tumors expressing ER and/or progesterone receptor (PR) are considered hormone receptor- positive breast cancers, whereas tumors that do not express ER, PR or HER2 are triple- negative breast cancer (TNBC) (Mamounas *et al.,* 2018; Nasser *et al.*, 2015). Although many types of cancers including breast cancer are initially susceptible to anti-cancer drugs, tumor cells can develop resistance over time through different mechanisms that impair drug efficacy. The ability of cancer cells to become simultaneously resistant to different drugs—a trait known as multidrug resistance (MDR)—remains a significant impediment to successful chemotherapy (Cai *et al.*, 2018; Wijdeven *et al.,* 2016);with efflux of drugs as one of the cause of drug resistance (Tsou *et al.*, 2015). Doxorubicin (Dox) is part of anthracyclin family and is chemotherapeutic drug used to treat breast cancer. It has however been shown that drug

resistance result in poor patients prognosis and survival. Although several mechanisms have been investigated, Dox resistance still remains a major unsolved issue in the treatment of breast cancer patients. Chemoresistance limits the efficacy of anticancer therapies and negatively impacts the long-term survival of cancer patients. It is crucial to unravel the mechanisms underlying the acquisition of drug resistance during treatment to facilitate the development of novel therapeutic strategies for eradicating breast cancer relapses (Dong *et.al.,* 2020). Small extracellular vesicles (sEVs)/exosomes are heterogeneous populations of small membrane vesicles consist of lipid bilayer membrane that are released by cells, carrying a variety of functional nucleic acids, proteins, and lipid cargos important in cell–cell communication. Recently, it has become apparent that secreted extracellular vesicles (EVs)/exosomes are proficient intercellular communication mediators (Sullivan *et.al.*, 2017). Small extracellular vesicles/exosomal cargos, microRNA (miRNA), lncRNAs, and circRNAs have various functions in cancers including cell proliferation, migration, invasion and metastasis. Envisioning and understanding the potential molecular mechanisms responsible for drug resistance and to find role of sEVs/exosomes associated gene signature with drug resistance hold a great potential for a more effective BC treatment. Taken together, these findings promote us to investigate whether sEVs/exosomes derived from resistant cells could mediate drug resistance in breast cancer cells. Mastering our understanding of these resistance mechanisms will help in improving breast cancer treatments, and the concept of tumour-secreted factors, such as sEVs/exosomes, as a means of communication within the metastatic process

Hypothesis

Keeping these findings in view, we hypothesize that doxorubicin resistant breast cancer cells secrete more small extracellular vesicles/exosomes with protein signature that

communicate with non resistant cells and may confer drug resistance by deregulating pathways in nonresistant breast cancer cells.



Aim& Objectives

The present study has been designed to evaluate the role of exosomes in drug resistance in breast cancer and elucidiating the key proteins, signaling pathways and their mechanism of action in inducing doxorubicin drug resistance

Objectives

The present study is being undertaken with the following specific objectives in view.

1. Isolation and quantification of sEVs/Exosomes secreted from resistant and non resistant breast cancer cells .
2. Role of sEVs/Exosomes released from resistant cells in inducing resistance in breast cancer cells.
3. Determine deregulated protein signature associated with sEVs/Exosomes in drug resistance.

4. To identify molecular pathway(s) and mechanism of action of deregulated protein(s) in drug resistance.

Review of Literature:

International status

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer-related death in females worldwide (Rebecca *et al.,* 2022). Breast cancer (malignant breast neoplasm) is cancer originating in the breast tissue that is made up of glands for milk production, called lobules, and the ducts that connect the lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissues (Kozma *et al.,* 2021;). Breast cancer is a variegated disease with over 20 subtypes that differ in anatomy, genetics, and clinical behavior (Jenkins *et al.,* 2021). Clinically, conventional drugs, such as chemotherapeutic, hormonal, and targeted drugs, remain the first-line drug treatment (Dong *et al.,* 2020). BC cells can employ a variety of mechanisms to escape cell death induced by chemotherapeutic drugs (Fig 1)(Jayaraj *et al.,* 2019). These mechanisms mainly include the efflux and inactivation of drugs (Dallavalle *et al*., 2020), activation of bypass signaling or prosurvival pathways, enhancement of DNA damage repair (Battista *et al*., 2020), and induction of epithelial–mesenchymal transition (EMT)(Nayas *et al.*, 2020) and stem-like property (Garcia *et al*., 2020) . The study of extracellular vesicles (EVs) has rapidly expanded in the past decade. EVs are a heterogeneous population of cell-derived membrane vesicles that are secreted by various cell types. They exhibit a wide size

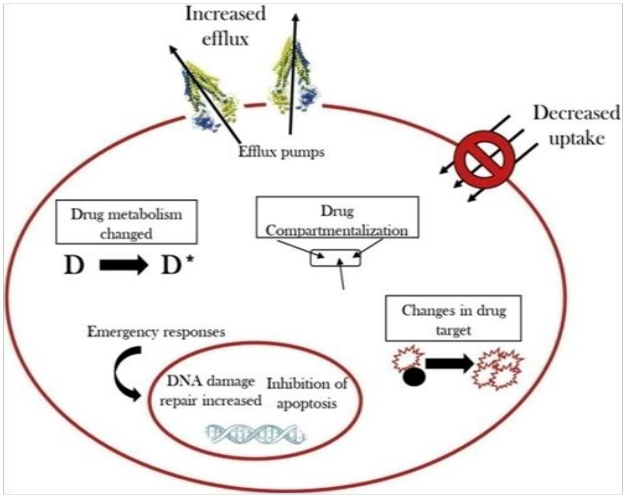


Fig 1) Adapted from Drug Resistance Updates, 2020 volume 50:100682)

range and differ by their biogenesis. EVs include exosomes, which are small membrane vesicles, ranging from 30 to 150 nm in diameter, and shed microvesicles (MVs), which are large membrane vesicles of 150 to 1000 nm diameter budding off the plasma membrane (Van *et al.,* 2018) . Smaller MVs have also been reported, which are ~ 100 nm in diameter (Nabhan *et al*., 2012) . EVs carrying a variety of functional nucleic acids, proteins, and lipid cargos important in cell–cell communication (Maacha *et al.,* 2019). Bases on size and characteristics EVs consists of three major subsets of vesicles called as exosomes, microvesicles and apoptotic bodies .All eukaryotic cells stay in contact with their local environment by transporting biological material across membranes via the secretion of small vesicles called "exosomes" that are endocytic in origin (40–100 nm in size). These are found in body fluids, including amniotic fluid, ascites, bile, breast milk, nasal and bronchial lavage fluid, blood plasma, saliva, semen, synovial fluid, and urine, allowing extraction of EVs from various liquid biopsies (Matei *et al.,* 2017). EVs contain proteins, lipids, DNA, RNA, and micro RNA serving as mediators of cell-to-cell communication (Mao *et al.,* 2019). Besides tetraspanins, other molecules are also found on the surface of exosomes, e.g., ALIX, TSG101, HSP70, flotillin, LAMP-1, MHC-I, -II. The biogenesis of EVs oocur when endocytosis of the cell membrane form early endosomes and early endosomes encapsulate specific substances to mature into multivesicular bodies (MVBs), whose formation is mainly regulated by the endosomal sorting complex required for transport (ESCRT) which can recognize ubiquitinated modified membrane proteins and promotes their degradation. One of the best-characterized mechanism of exosome biogenesis involves the recruitment of the endosomal sorting complex required for transport (ESCRT) machinery to ubiquitinated proteins in the early endosome. There are four ESCRT complexes (ESCRT-0, −I, −II, and -III), which associate with ESCRT-associated accessory proteins, such as the ATPase VPS4, its cofactor VTA-1, TSG101, and Alix. ESCRT-0 complex recognizes and sequesters

ubiquitinated proteins on the outside of the endosomal membrane. ESCRT-I and –II complexes are responsible for starting and driving intraluminal membrane budding. ESCRT-III complex performs vesicle scission to form Multivesicular bodies (Abels *et al.,*

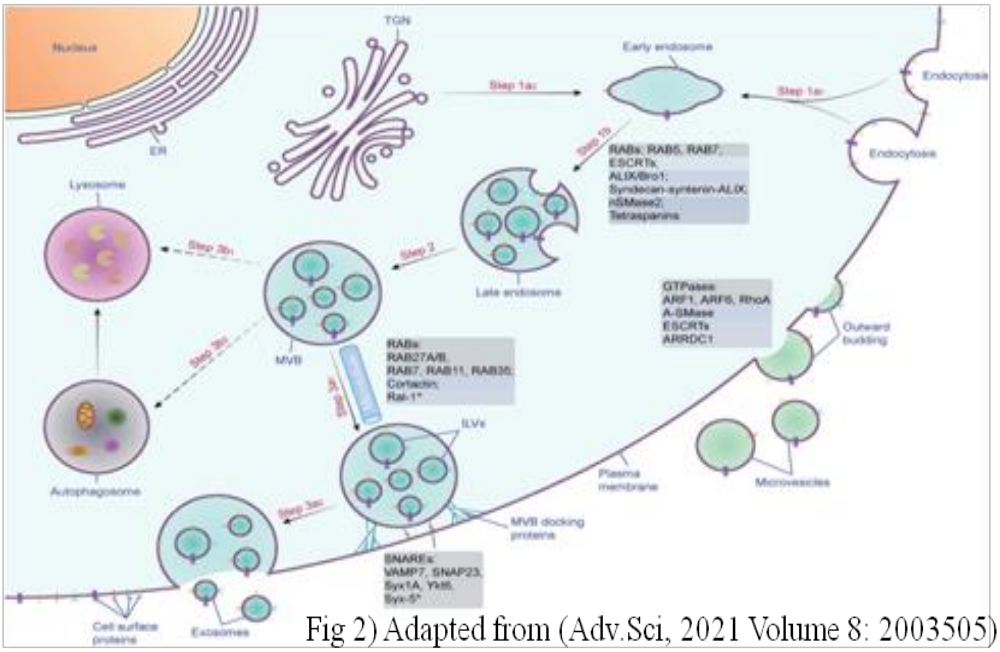


Fig 2) Adapted from (Adv.Sci, 2021 Volume 8: 2003505)

2016). Trajkovic et al. have also described an ESCRT-independent exosome biogenesis pathway, which is mediated by the sphingolipid ceramide. Following the secretory pathway, exosomes merge with the plasma membrane and are released into the extracellular milieu or fused with lysosomes for degradation (Fig 2) (Beach *et al.,* 2014).

Exosomes are of particular interest in biological research because their unique biogenesis involves distinct intracellular regulatory processes that give them specific cargos and thus different biological functions. BC is a highly heterogeneous disease comprised of multiple distinct subtypes, thus exosomes mediate drug resistance through different mechanisms. The contents, membrane composition and size of EVs are highly heterogenous and depend on cellular location and environment. EVs are potent vehicles of intercellular communication providing protection and maintenance for cells and regulating cellular functions (Yanez *et al.,* 2015). They are involved in cell-to cell communication, immune response, angiogenesis,

and signal transduction. EVs mediate intercellular communication by transferring proteins and nucleic acids to remote target cells. The development of drug resistance via EVs is articulated around mechanisms involving such cargo. EVs can mediate drug resistance by directly exporting or sequestering cytotoxic drugs, reducing their effective concentration at target sites. Recent evidence has shown that EVs play an important role not only in mediating drug resistance, but also in conferring resistance to drug-sensitive cancer cells. EVs are capable of horizontal transfer of specific bioactive cargoes that may alter cell cycle control and apoptotic programs in recipient cells. EVs can also mediate intercommunication between cancer cells and stromal cells within the tumor microenvironment, leading to the acquisition of drug resistance and tumor progression. A critical biological feature that contributes significantly to cancer progression, invasion and metastasis is the 'tumor microenvironment'(Zhang *et al.,* 2021). The tumor microenvironment (TME) is an interactive cellular environment surrounding the tumor whose main function is to establish cellular communication pathways supporting tumorigenesis (Eguchi *et al.,* 2022). The cellular component of the TME mainly comprises immune and inflammatory cells, stromal fibroblasts, and endothelial cells forming the blood vessels that secrete a series of extracellular/angiogenesis signaling molecules, which in turn lead to a functional modulation of TME (Fu *et al.,* 2016). The extracellular vesicles (EVs) secreted by tumors, commonly known as tumor-derived EVs, have been well documented to modulate the tumor microenvironment. EVs plays prominent role in formation of premetastatic niche by conducive environment for metastasis which includes stromal activation, conversion of fibroblasts to myofibroblasts and modulation of ECM (Baglio *et al.,* 2017). Thus, EVs are highly specialized entities of communication carrying several surface markers and signaling molecules, oncogenic proteins and nucleic acids that can be transferred horizontally to the stromal target cells and condition the tumor microenvironment for an improved tumor

growth, invasion, and metastasis (Yu *et al.,* 2016). EVs have been shown to be critical mediators of the immune-cancer cell communication. Exosomes possess ability to disable cytotoxic arm of immune response by inducing apoptosis in cytotoxic T-cells and reducing proliferation of NK cells (Maybruck *et.al.,*2017). Cancer cells are known to export drugs in the extracellular space using specialized transporters of the multi-drug resistance (MDR)-ATP binding-cassette (ABC transporters system (Robey *et al.,* 2018). These pumps reduce the intracellular accumulation of many anti-cancer drugs to sub-therapeutic levels, thus decreasing or abolishing drug efficacy. The emergence of EVs as drug resistant modulators has added to the complexity of resistance mechanism. In addition, EVs can be utilized by cancer cells as drug vehicles to facilitate drug resistance through drug sequestration and expulsion (Selma *et al.,* 2019) EVs mediate intercellular communication by transferring proteins and nucleic acids to remote target cells. The development of drug resistance via EVs is articulated around mechanisms involving such cargo. EVs can mediate drug resistance by directly exporting or sequestering cytotoxic drugs, reducing their effective concentration at target sites. Extracellular vesicles-mediated intercellular transfer of functional MRP1 drug efflux transporter (ABCC1) was reported in leukemia cells (Console *et al*., 2022). Selective P-gp/MDR-1 mRNA in EVs released from doxorubicin-resistant osteosarcoma cells suggests that resistant tumor cells use several means to spread drug resistance to sensitive cells, either by transferring MDR proteins directly to sensitive cells or by transferring the mRNA that encodes them, contributing to the diversity of drug resistance mechanisms. Exosomes are effective in transferring drug resistance as well as P-gp from drug-resistant breast cancer cells to sensitive ones (Zhang *et al*., 2014). Meanwhile, exosomal cargos, especially ncRNAs, such as microRNA (miRNA), lncRNAs, and circRNAs have various functions in cancers. By regulating target mRNA, miRNA affects many vital cellular processes and the development of cancer cells, such as cell proliferation,

migration, invasion and metastasis. MiRNAs are short RNA molecules that regulate mRNA expression post-transcriptionally by post-transcriptional gene silencing. Exosomal miRNA plays a critical role in cancers by regulating the stability of target mRNA or inhibiting translation (Kim *et al.,* 2012) . Several studies have shown that miRNA let-7a can act as a tumor suppressor by regulating the cell cycle and cellular differentiation . Exosomal Let-7a binding on the 3′ UTR region of the c-Myc gene and silencing its expression, which inhibited proliferation, migration, and invasion of MDA-MB-231 cell (Tang *et al*., 2022). lncRNA MALAT1 was over-expressed in breast cancer cells and their exosomes, and significantly increased the proliferation of TNBC cells (Zhang *et al.,* 2018). Exosomal miR-10b is reported to promote cell migration, metastasis and invasion of breast cancer. MiR-10b is highly expressed in metastatic breast cancer MDA-MB-231 cells and can be actively secreted into the extracellular via exosomes. Additionally, exosomal miR-10b can be absorbed by different cell lines and suppress the protein level of its target genes such as HOXD10 and KLF4 to promote cell invasion in breast cancer (Tang *et al*., 2022). HIF-1α-stabilizing long non-coding RNA (HISLA) packaged in tumor-associated macrophage (TAM) exosomes has been discovered and revealed as an essential oncogene that enhances aerobic glycolysis and apoptotic resistance in MDA-MB-231 cells (Benedetti *et al.,* 2022). Extensive reports have illustrated that exosomes derived from tumors are significantly involved in the modulation of the biological activities of their recipient cells via the transfer of their oncogenic content. For example, exosomes play a role in the induction of normal cell transformation. A study has demonstrated that prostate cancer cell-derived exosomes are involved in the clonal expansion of tumors through reprogramming of adipose-derived stem cells via trafficking of oncogenic factors such as H-ras and K-ras transcripts, as well as oncogenic miRNAs miR-125b, miR-130b, and miR-155 (Abd *et al.,* 2014). It was determined that exosomes derived from cells and sera of breast cancer patients was able to

promote the formation of tumors from non tumorigenic epithelial cells in a Dicer-dependent manner (Hao et al., 2022) . T leukemia cells-derived exosomes was able to transport miRNAs (miR92a) to endothelial cells to modulate endothelial migration and tube formation (Yadav *et al.,* 2022). Thus there is potential for the development of exosomes as diagnostics biomarkers as they contain all the bioactive molecules of the cells they are derived from.

National status

With annual percentage change of 0.4-0.6 in incidence, breast cancer in India emerged as most common cancer in women over last two decades with 26.3% prevalence (Asthana *et al.,* 2014). TNBC is reported higher in India than those of western women that accounts for 27% of total BC. Treatment with chemotherapeutic drugs increases the expression of ABC transporters. Inhibitors of ABC transporters lead to chemosensitization of these and improved treatment outcome (Saxena *et al.,* 2011). Exosomes export upstream signaling pathway components in cervical cancer cells which are responsible for cervical cancer progression and tumor microenvironment conditioning (Sharma *et al*., 2018). Role of exosomes in mediating increments in miR-155 expression in oral squamous carcinoma cells that have acquired cisplatin resistance (cis^{Res} cells). exosomal transfer from cis^{Res} to the cisplatin sensitive (cis^{Sens}) cells was found to confer significant miR-155 induction in the recipient cis^{Sens} cells. Restoration of miR-155 expression in cis^{Sens} cells following miR-155 mimics treatment led to epithelial to mesenchymal transition, enhancements in their migratory potential as well as acquisition of resistant phenotype (Kiraye *et al.,* 2020). Exosome-mediated delivery of miR-30a sensitize cisplatin-resistant variant of oral squamous carcinoma cells via modulating Beclin1 and Bcl2. Beclin1, an autophagy-related marker, as a target of miR-30a was found to be overexpressed in cisplatin-resistant oral squamous cell carcinoma cells, thus indicating at its possible negative-regulation by miR30a (Kulkami *et*

al., 2020). Not much work has been done in our country on exosomal transfer of Chemoresistance. Studies have focused on exosomal microRNAs transfer in various cancers. However, the mechanisms by which proteins in sEVs/exosomes affect the phenotype of recipient cells have not been fully interpreted, especially in chemotherapy resistance. Understanding the potential molecular mechanisms responsible for drug resistance and to find role of sEVs/exosomes for a more effective BC treatment. Taken together, these findings promote us to investigate whether sEVs/exosomes derived from resistant cells could mediate drug resistance in breast cancer cells.

Identification of Research Gaps

Breast cancer (BC) is the most prevalent malignancy and the leading cause of cancer-related death in women. Despite the chemotherapy BC is still being confronted with high invasion, metastasis, recurrence rate, and drug resistance. It is a well established fact that chemotherapeutic drugs employ a variety of mechanisms that impart resistance, thus making Chemoresistance a major challenge for successful anticancer therapy. As a result there is shorter survival of the breast cancer patients. In order to improve the disease free survival of breast cancer patients understanding of the factors that contribute to breast cancer resistance need to be studied. Small EVs are known to transport biological material and play role in the development and progression of cancer. These cell contents, when transferred to recipient cells, and alter biological behavior. However the Studies till now, have focused on exosomal microRNAs transfer in various cancers. The mechanisms by which proteins in sEVs/exosomes affect the phenotype of recipient cells have not been fully interpreted, especially in chemotherapy resistance. In this work, understanding the potential molecular mechanism responsible for drug resistance and to find role of sEVs/exosomes and elucidating the related mechanism may possibly address the problems like drug resistance in breast cancer malignancy. This work will provide a deeper insight in understanding the

molecular mode of action of exosomes and its role governing resistance, for a more effective BC treatment. Taken together, these findings promote us to investigate whether sEVs/exosomes derived from resistant cells could mediate drug resistance in breast cancer cells. Therefore, the study may prove necessary to optimize therapeutic interventions for better clinical outcome and improved patient survival.

Methodology

Objective 1: Isolation and quantification of sEVs/Exosomes secreted from resistant and non resistant breast cancer cells .

Approach: One of the key goals of this study is to understand the characteristics and insights into the drug resistance so we will develop *in vitro* breast cancer model in (MCF-7, MDA-MB-231,MDA-MB-468) cell lines. The exosomes will be isolated from resistant and non resistant breast cancer cell lines using differential ultracentrifugation method. The nanoscale visualization of the exosomes from the two models of breast cancer that will give clear distinction between the morphology and physiological status of exosomes isolated from the two types of cells (resistant and non resistant breast cancer cells) that will be confirmed by transmission electron microscopy. We will identify the markers as characteristics of sEVs/exosomes by western blotting using specific antibodies. Proper quantification of exosomes will be done using FACS.

- Cell Culture
- Development of resistant breast cancer cells
- Ultracentrifugation
- Transmission electron microscopy
- Western blot
- Fluorescence-activated cell sorting

1.1 a) Cell Culture

Breast cancer cell lines will be maintained at 37ºC and 5% CO_2 in a humidified atmosphere. Cells will be grown in Dulbecco's modified Eagle's medium supplemented with 0.01 mg/ml insulin and 500 ng/ml hydrocortisone. Media for all cell lines will be supplemented with 10% FBS, 50 U/ml penicillin and 5 mg/ml streptomycin (Reyes et al., 2017).

b) Development of Resistant breast cancer cell line

Human breast cancer cell lines will be cultured in standard conditions. Then we will develop resistant cell lines from parental breast cancer cell lines cells using a stepwise increase in treatment dose with doxorubicin (Dox). This will induce greater fold resistance in cell than that observed in the parental cells. The sensitivity of these paired cell lines will be determined by exposing daughter cell to range of drug concentrations (Dox) and no treatment will be given to parental cell line and then cell viability will be assessed. The IC_{50} (drug concentration causing 50% growth inhibition) for these cell lines will then be used to determine the increase in resistance. The resistant daughter cells will then be compared to the parental cells using cell viability assays such as the MTT (Nasser *et al.,* 2015).

1.2) Exosome isolation from resistant and non resistant breast cancer cells

Exosomes will be isolated from culture medium of both resistant and non resistant cells. Supernatant will be centrifuged at 400 ×g for 10 min at 4 °C and then again centrifuged at 10,000×g for 30 min at 4 °C to remove apoptotic bodies. Supernatant from the former step will be filtered through 0.22µm porous membrane followed by centrifugation at 100,000×g at 4ºC for 70 min using an ultracentrifuge. Exosomes pellet will be washed in PBS, ultracentrifuged again and suspended in phosphate buffer saline (PBS).

1.3) Visual confirmation of size and morphology of sEVs/Exosomes

sEVs/Exosomes will be visualized by Transmission electron microscopy (TEM). Exosomes will be suspended in PBS and placed onto copper grids at room temperature. Extra solution will be removed with filter paper, and phosphotungstic acid solution will be added. Then Filter paper will be used again to remove the excess solution. The copper grids will be dried at room temperature for 2 min, and exosomes will be observed transmission electron microscopy**.**

1.4) Characterization of sEVs/Exosomes will be visualized by Western blot

sEVs/Exosomes extracts will be lysed with RIPA buffer containing protease inhibitor protein samples will be loaded and run on SDS-PAGE gels and transferred to Hybridization Nitrocellulose Filter. Then, the membranes will be blocked with 5% skimmed milk dissolved in 0.1% Tris-buffered saline with Tween-20 (0.1% TBST) at room temperature for 1 h. Next, the membranes will be incubated with primary antibodies diluted in primary antibody solution (TSG101, CD63) overnight at 4 °C. On the following day, 0.1% TBST will be used to wash the membranes. Then, the membranes will be incubated with secondary antibodies (Anti TSG101, CD63) diluted in 0.1% TBST at room temperature for 2 h. Signals will be developed with ECL and protein bands will be visualized.

Quantification of exosomes will be done by

1.5) Flourescent activated cell sorting (FACS)

Cell suspension of resistant and non-resistant exosomes will be subjected to FACS Antibody specific to CD63, TSG101 associated with fluorescent molecule will be tagged and added to mixture of cells and will be detected through flow cytometer.

Methodology of Objective 2

Role of sEVs/Exosomes released from resistant cells in inducing resistance in breast cancer cells

Approach: Breast cancer malignancies become resistant to drug over time. To analyze exosome function, we will show exosomes secreted by chemoresistant cancer cells are subsequently taken up by recipient cells render those recipient cells more apt to resistance which will be assessed by cellular proliferation analysis that is MTT assay. Flow cytometry analysis will shed light on how sEVs/exosome is responsible for stimulating proliferation in non resistant cells, treated with exosomes from resistant cells and will have capacity to evade apoptosis and survive even when higher doses of DXR is given. Common surface protein markers that will be highly expressed on exosomes from resistant breast cancer cells than non resistant ones, include CD81 and TSG101. Dysregulation in cell proliferation could be assessed using PCNA, with an increase in PCNA immunoreactivity associated with an increase in cell proliferation. Regulation of exosomes by EMT in inducing resistance in non- resistant breast cancer cells will be checked by markers of EMT using western blot

- Cell viability Assay
- Flow cytometry
- Western blot

2.1) Cell viability Assay

In order to test cell growth viability non resistant breast cancer cells will be treated with sEVs/exosomes from resistant breast cancer cells .Cells will be seeded into 96-well plates, with growth medium for a well. After cultured for 72 h, chemosensitivity will be assessed. The value will be used as an indicator of cell viability.. Finally, the absorbance will be measured at 570 nm using a 96 well microplate reader. The cytotoxicity index will be determined using the untreated cells as negative control. The IC_{50} will be extrapolated from

the dose-response graph. The compound concentration that reduces the viability of cells by 50% (IC_{50}) will be determined by plotting quadruplicate data points over a concentration range. Experiment will be repeated at least 3 times.

2.2) Flow cytometry

Apoptosis will be checked after 72 h and the cells will be harvested according to the manufacturer's instructions using an Annexin V-APC/7-AAD staining kit. The apoptotic rate of the cells will be determined by flow cytometry .Cell cycle assays will be performed after 24 h, and cells will be harvested according to the manufacturer's instructions for the cell cycle staining buffer.

2.3) For checking expression of various markers determining the cancer progression

In order to determine the cancer progression various proliferating markers like PCNA (Proliferating cell nuclear antigen) expression will be checked by Western Blot. Markers of epithelial mesenchymal transition (EMT) will be evaluated. Expression of epithelial markers like E-cadherin, Catenin and mesenchymal markers like N- cadherin, Fibronectin, Vimentin will be checked by Western Blot by using specific antibodies.

Methodology of Objective 3

Determine deregulated protein signature associated with sEVs/Exosomes in drug resistance

Approach: Phospho-protein arrays will be useful for the consideration of druggable molecular targets within a specific tumor. Analyses of the deregulated proteins associated with exosomes in chemoresistant breast cancer cell, phosphorylation profiles of a broad spectrum of receptor tyrosine kinases and their downstream signaling proteins will be performed by the Proteome Profiler of Human Phospho-RTK Array Kit. To further evaluate

the effect of Chemoresistance on the kinase profile, we will analyze the phosphorylation status of several kinases. To address the mechanism underlying the alterations by exosomes in recipient cells, we will obtain a proteomic profile of chemoresistant-derived exosomes that will show expression changes favoring a resistant/proliferative profile. Thus, We will find out a common pattern for these cell lines the phosphorylated presence of key signalling nodes. Alternate strategies will be used if the relative levels of expression or phosphorylation of several proteins will not be detected .Then the total protein analysis will be done by mass spectrometry. Identification of proteins involved in exosomal transfer of breast cancer capable of promoting Chemoresistance will be analyzed.

- Phospho Kinase Array
- Mass Spectometry

3.1) Phospho Kinase Array

To detect relative site specific Phosphorylation of proteins from exosomes will be done by Phospho-Kinase Antibody Array. Selected captured antibodies spotted on nitrocellulose membrane will bind to specific protein present on target. Cell lysate samples will be diluted and mixed with biotinylated detection antibody. Sample/antibody mixture will be then incubated with the array. Then will be visualized using chemiluminescent detection reagents. Signal produced will be proportional to amount in bound analyte.

3.2) Mass spectometry

a)Exosomal Protein Lysis and Digestion and peptide fraction will be done

Exosomes from the resistant and non resistant cells will be solubilized in lysis buffer. Protein concentration will be determined Protein from the two groups will be reduced with

dithiothreitol and alkylated with iodoacetamide. Peptides will be fractionated by high pH reverse-phase HPLC.

b) Liquid Chromatography Coupled to Tandem Mass Spectrometry

Peptides will be dissolved and separated by an ultra-high-performance liquid system. After separation, the peptides will be injected into the NSI source for ionization and then analyzation. Electrospray voltage will be set at 2.0 kV, scanning range for primary mass spectrometry was set to 350–1,550 m/z. Scanning range for secondary mass spectrometry will 100 m/z. Data acquisition mode will use a data-dependent scanning (DDA) program.

c) Data Processing

Raw MS/MS data will be processed using the MaxQuant search engine. The tandem mass spectra will be queried against the Human UniProt/SwissProt database combined with the reverse decoy database. Mass tolerance value for precursor ions will be set for the first search and the main search. Proteins will be quantified using label-free quantification. The differential expression threshold will be set

d) Protein identification

Bioinformatics Analysis

Subcellular localization of all identified proteins will be predicted by wolfpsort (protein subcellular localization prediction tool). Gene Ontology (GO) enrichment analysis, proteins will be classified by GO annotation. Three categories biological process, cellular compartment, and molecular function will be find out. Fisher exact test will be performed to compare the enrichment of the differentially expressed proteins to that of all identified proteins.

Methodology of Objective 4

To identify molecular pathway(s) and mechanism of action of deregulated protein(s) in drug resistance

Approach: The mechanism of exosomal release by the tumor cells and its contribution to tumor metastasis and drug resistance likely play a role in breast cancer. Investigating the role of protein involved exosomal transfer from resistant to sensitive breast cancer cell and the identification of exosomal protein involved in resistance and the analysis of its verified function in doxorubicin resistance will be done. Thus we will find the unconventional mechanism of drug resistance in breast cancer. Further, identification of the signaling pathway involved in the transfer of exosomal protein by the bioinformatic approach (KEGG) will be detected that will help us to find the emerging evidence that breast cancer derived exosome may contribute to recruitment and reprogramming of non resistant breast cancer cell. The interaction of the proteins with the other downstream proteins will be done by protein-protein interaction network analysis. The functional mechanism of action will be inferred by the downregulation or upregulation of the key protein by siRNA transfection.

- Kyoto Encyclopedia of Genes and Genomes
- Search tool for retrieval of interacting genes/proteins
- Protein-protein interaction network (PPI) analysis
- siRNA transfection

4.1) Functional Pathway enrichment analyses

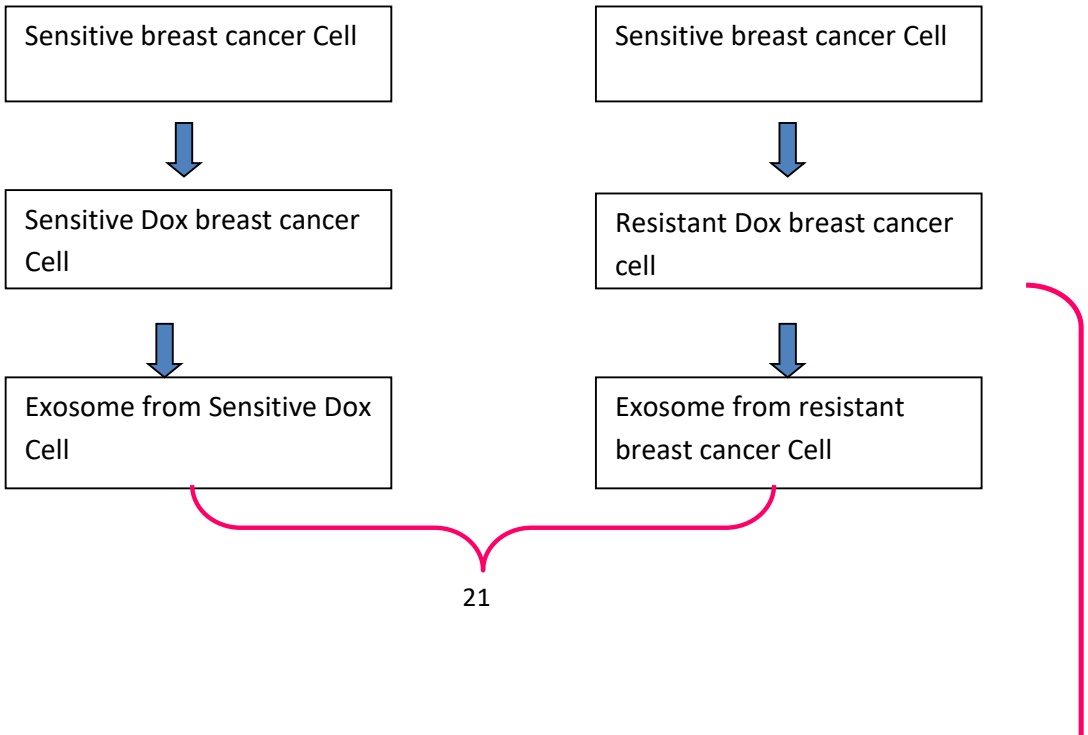
Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) will be used to identify the enriched pathways. Fisher's exact test will be used to compare the enrichment of the differentially expressed proteins to that of all identified proteins

4.2) Interaction of deregulated proteins will be seen by online database. STRING (Search tool for retrieval of interacting genes/proteins) database will be used for protein-protein interaction network (PPI) analysis. Only interactions between proteins included in the data search set will be selected, thereby excluding external candidates. Interaction network generated by using STRING will be visualized in Cytoscape. CytoHubba plug-in of Cytoscape software will be used to select the essential hub proteins among the DEPs. Analysis results will be determined according to the maximal clique centrality (MCC) method .In order to find out the mechanism of action of deregulated proteins and the pathways involved we will do the overexpression and downregulatin of the key proteins by

4.3) siRNA transfection

Chemically synthesized siRNAs and control siRNAs will be purchased. The siRNAs will be transiently transfected into resistant and non resistant cells using Lipofectamine according to the manufacturer's instructions. After 48 h of transfection, the cells will be analyzed by WB, and the conditional medium will be collected**.**

Work flowchart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Resistant Dox breast cancer cell
Exosome from Sensitive Dox Cell
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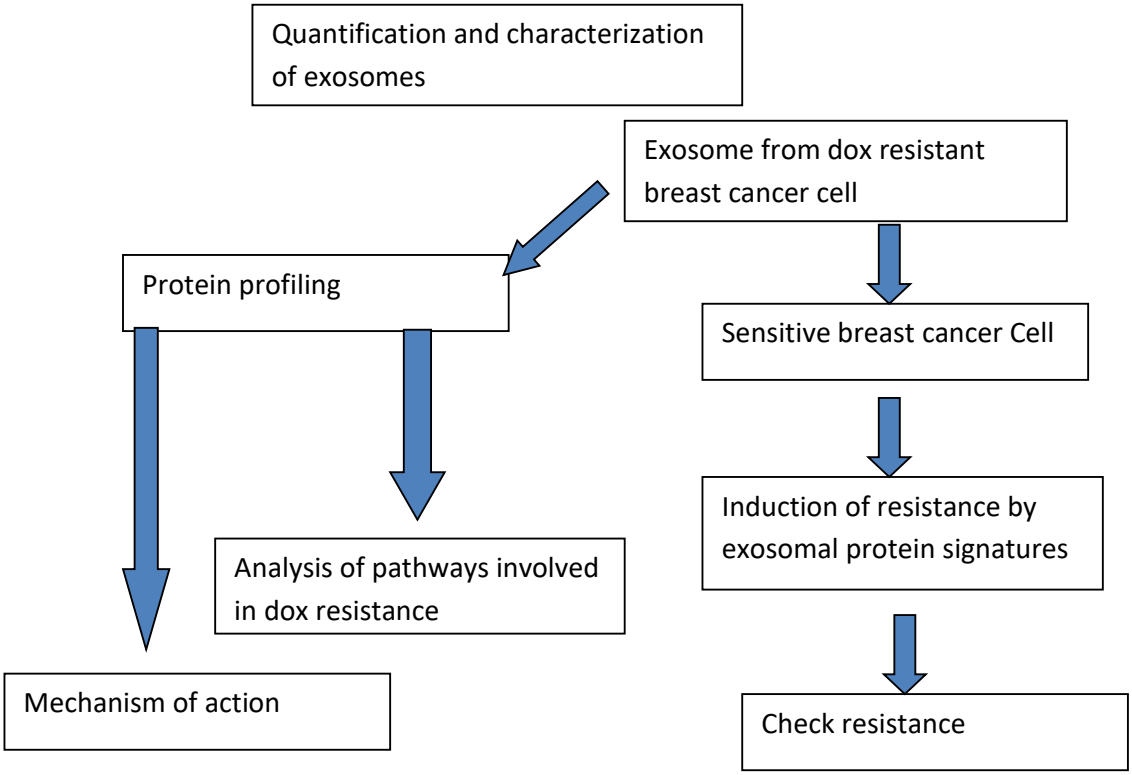
Quantification and characterization of exosomes
Exosome from dox resistant breast cancer cell
Protein profiling
Sensitive breast cancer Cell
Induction of resistance by exosomal protein signatures
Analysis of pathways involved in dox resistance
Mechanism of action
Check resistance

References

Abd Elmageed, Z. Y., Yang, Y., Thomas, R., Ranjan, M., Mondal, D., Moroz, K., ... & Abdel-Mageed, A. B. (2014). Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes. *Stem cells*, *32*(4), 983-997.

Abels, E. R., & Breakefield, X. O. (2016). Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. *Cellular and molecular neurobiology*, *36*(3), 301-312.

Asthana, S., Chauhan, S., & Labani, S. (2014). Breast and cervical cancer risk in India: an update. *Indian journal of public health*, *58*(1), 5.

Battista, T., Fiorillo, A., Chiarini, V., Genovese, I., Ilari, A., & Colotti, G. (2020). Roles of Sorcin in drug resistance in cancer: one protein, many mechanisms, for a novel potential anticancer drug target. *Cancers*, *12*(4), 887.

Beach, A., Zhang, H. G., Ratajczak, M. Z., & Kakar, S. S. (2014). Exosomes: an overview of biogenesis, composition and role in ovarian cancer. *Journal of ovarian research*, *7*(1), 1-11.

Benedetti, A., Turco, C., Fontemaggi, G., & Fazi, F. (2022). Non-coding RNAs in the crosstalk between breast cancer cells and tumor-associated macrophages. Non-coding RNA, 8(1), 16

Bhat, A., Sharma, A., & Bharti, A. C. (2018). Upstream Hedgehog signaling components are exported in exosomes of cervical cancer cell lines. *Nanomedicine*, *13*(17), 2127-2138.

Cai, Y., Zheng, Y., Gu, J., Wang, S., Wang, N., Yang, B., ... & Wang, Z. (2018). Betulinic acid chemosensitizes breast cancer by triggering ER stress-mediated apoptosis by directly targeting GRP78. *Cell death & disease*, *9*(6), 1-16.

Console, L., & Scalise, M. (2022). Extracellular Vesicles and Cell Pathways Involved in Cancer Chemoresistance. Life, 12(5), 618.

Dallavalle, S., Dobričić, V., Lazzarato, L., Gazzano, E., Machuqueiro, M., Pajeva, I., ... & Fruttero, R. (2020). Improvement of conventional anti-cancer drugs as new tools against multidrug resistant tumors. *Drug Resistance Updates*, *50*, 100682.

Dong, X., Bai, X., Ni, J., Zhang, H., Duan, W., Graham, P., & Li, Y. (2020). Exosomes and breast cancer drug resistance. *Cell death & disease*, *11*(11), 1-14.

Eguchi, T., Sheta, M., Fujii, M., & Calderwood, S. K. (2022, January). Cancer extracellular vesicles, tumoroid models, and tumor microenvironment. In Seminars in cancer biology. Academic Press.

Ferlay, J., Ervik, M., Lam, F., Colombet, M., Mery, L., Piñeros, M., ... & Bray, F. (2018). Global cancer Observatory: cancer today. Lyon, France: international agency for research on cancer.

Fu, H., Yang, H., Zhang, X., & Xu, W. (2016). The emerging roles of exosomes in tumor–stroma interaction. *Journal of cancer research and clinical oncology*, *142*(9), 1897-1907.

Garcia-Mayea, Y., Mir, C., Masson, F., Paciucci, R., & LLeonart, M. E. (2020, February).Insights into new mechanisms and models of cancer stem cell multidrug resistance. In *Seminars in cancer biology* (Vol. 60, pp. 166-180). Academic Press.

Hao, Q., Wu, Y., Wu, Y., Wang, P., & Vadgama, J. V. (2022). Tumor-Derived Exosomes in Tumor-Induced Immune Suppression. *International Journal of Molecular Sciences*, *23*(3), 1461.

Jenkins, S., Kachur, M. E., Rechache, K., Wells, J. M., & Lipkowitz, S. (2021). Rare breast cancer subtypes. *Current oncology reports*, *23*(5), 1-14.

Kim, S. J., Shin, J. Y., Lee, K. D., Bae, Y. K., Sung, K. W., Nam, S. J., & Chun, K. H. (2012). MicroRNA let-7a suppresses breast cancer cell migration and invasion through downregulation of CC chemokine receptor type 7. *Breast Cancer Research*, *14*(1), 1-12.

Kirave P, Gondaliya P, Kulkarni B (2020 March).Exosome mediated miR-155 delivery confers cisplatin chemoresistance in oral cancer cells via epithelial-mesenchymal transition. *Oncotarget*. 27531 11(13):1157-1171.

Kulkarni, B., Gondaliya, P., Kirave, P., Rawal, R., Jain, A., Garg, R., & Kalia, K. (2020). Exosome-mediated delivery of miR-30a sensitize cisplatin-resistant variant of oral squamous carcinoma cells via modulating Beclin1 and Bcl2. *Oncotarget*, 27557 *11*(20), 1832–1845.

Maacha, S., Bhat, A. A., Jimenez, L., Raza, A., Haris, M., Uddin, S., & Grivel, J. C. (2019). Extracellular vesicles-mediated intercellular communication: roles in the tumor microenvironment and anti-cancer drug resistance. *Molecular cancer*, *18*(1), 1-16.

Mamounas, E. P., Russell, C. A., Lau, A., Turner, M. P., & Albain, K. S. (2018). Clinical relevance of the 21-gene Recurrence Score® assay in treatment decisions for patients with node-positive breast cancer in the genomic era. *NPJ breast cancer*, *4*(1), 1-6.

Mao, X., & Jin, F. (2019). The exosome and breast cancer cell plasticity. *OncoTargets and therapy*, *12*, 9817.

Matei, I., Kim, H. S., & Lyden, D. (2017). Unshielding exosomal RNA unleashes tumor growth and metastasis. *Cell*, *170*(2), 223-225.

Maybruck, B. T., Pfannenstiel, L. W., Diaz-Montero, M., & Gastman, B. R. (2017). Tumor-derived exosomes induce CD8+ T cell suppressors. *Journal for immunotherapy of cancer*, *5*(1), 1-15.

Nabhan, J. F., Hu, R., Oh, R. S., Cohen, S. N., & Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma

membrane by recruitment of TSG101 protein. *Proceedings of the National Academy of Sciences*, *109*(11), 4146-4151.

Nasser, M. W., Wani, N. A., Ahirwar, D. K., Powell, C. A., Ravi, J., Elbaz, M., Zhao, H., Padilla, L., Zhang, X., & Shilo, K. (2015). RAGE mediates S100A7-induced breast cancer growth and metastasis by modulating the tumor microenvironment. *Cancer research*, *75*(6), 974-985.

Navas, T., Kinders, R. J., Lawrence, S. M., Ferry-Galow, K. V., Borgel, S., Hollingshead, M. G., ... & Parchment, R. E. (2020). Clinical evolution of epithelial–mesenchymal transition in human carcinomas. *Cancer research*, *80*(2), 304-318.

Ren, J.-X., Gong, Y., Ling, H., Hu, X., & Shao, Z.-M. (2019). Racial/ethnic differences in the outcomes of patients with metastatic breast cancer: contributions of demographic, socioeconomic, tumor and metastatic characteristics. *Breast cancer research and treatment*, *173*(1), 225-237.

Robey, R. W., Pluchino, K. M., Hall, M. D., Fojo, A. T., Bates, S. E., & Gottesman, M. M. (2018). Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nature Reviews Cancer*, *18*(7), 452-464.

Saxena, M., Stephens, M. A., Pathak, H., & Rangarajan, A. (2011). Transcription factors that mediate epithelial–mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. *Cell death & disease*, *2*(7), e179-e179.

Selma Maacha1 , Ajaz A. Bhat1 , Lizandra Jimenez2 , Afsheen Raza3 , Mohammad Haris1,4, Shahab Uddin5 and Jean-Charles Grivel1 Molecular Cancer (2019) 18:55 Extracellular vesicles-mediated intercellular communication: roles in the tumor microenvironment and anti-cancer drug resistance.

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2022). Cancer statistics, 2022. *CA: a cancer journal for clinicians*.

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer Statistics, 2021]. *CA: a cancer journal for clinicians*, *71*(1), 7-33.

Sullivan, R., Maresh, G., Zhang, X., Salomon, C., Hooper, J., Margolin, D., & Li, L. (2017). The emerging roles of extracellular vesicles as communication vehicles within the tumor microenvironment and beyond. *Frontiers in Endocrinology*, *8*, 194.

Takashima, T., Mukai, H., Hara, F., Matsubara, N., Saito, T., Takano, T., ... & SELECT BC study group. (2016). Taxanes versus S-1 as the first-line chemotherapy for metastatic breast cancer (SELECT BC): an open-label, non-inferiority, randomised phase 3 trial. *The Lancet Oncology*, *17*(1), 90-98.

Tang, W., Xia, M., Liao, Y., Fang, Y., Wen, G., & Zhong, J. (2022). Exosomes in triple negative breast cancer: From bench to bedside. Cancer letters, 527, 1-9.

Tsou, S. H., Chen, T. M., Hsiao, H. T., & Chen, Y. H. (2015). A critical dose of doxorubicin is required to alter the gene expression profiles in MCF-7 cells acquiring multidrug resistance. *PloS one*, *10*(1), e0116747.

Van Niel, G., d'Angelo, G., & Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nature reviews Molecular cell biology*, *19*(4), 213-228.

Vasan, N., Baselga, J., & Hyman, D. M. (2019). A view on drug resistance in cancer. *Nature*, *575*(7782), 299-309.

Wijdeven, R. H., Pang, B., Assaraf, Y. G., & Neefjes, J. (2016). Old drugs, novel ways out: Drug resistance toward cytotoxic chemotherapeutics. *Drug Resistance Updates*, *28*, 65-81.

Yadav, J., Aggarwal, N., Chaudhary, A., Tripathi, T., Baruah, D., Chhakara, S., ... & Bharti, A. C. (2022). Role of Exosomes in Tumor Induced Neo-Angiogenesis.

Yáñez-Mó, M., Siljander, P. R. M., Andreu, Z., Bedina Zavec, A., Borràs, F. E., Buzas, E. I., ... & De Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. Journal of extracellular vesicles, 4(1), 27066.

Zhang, F. F., Zhu, Y. F., Zhao, Q. N., Yang, D. T., Dong, Y. P., Jiang, L., ... & Ma, X. (2014). Microvesicles mediate transfer of P-glycoprotein to paclitaxel-sensitive A2780 human ovarian cancer cells, conferring paclitaxel-resistance. *European journal of pharmacology*, *738*, 83-90.

Zhang, D. X., Vu, L. T., Ismail, N. N., Le, M. T., & Grimson, A. (2021, September). Landscape of extracellular vesicles in the tumour microenvironment: Interactions with stromal cells and with non-cell components, and impacts on metabolic reprogramming, horizontal transfer of neoplastic traits, and the emergence of therapeutic resistance. In Seminars in cancer biology (Vol. 74, pp. 24-44). Academic Press.

Zhang, P., Zhou, H., Lu, K., Lu, Y., Wang, Y., & Feng, T. (2018). Exosome-mediated delivery of MALAT1 induces cell proliferation in breast cancer. *OncoTargets and therapy*, *11*, 291.